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Light-Dependent Isoprene Emission[1]

Characterization of a Thylakoid-Bound Isoprene Synthase in *Salix discolor* Chloroplasts

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Isoprene synthase is an enzyme that is responsible for the production of the volatile C5 hydrocarbon, isoprene, in plant leaves. Isoprene formation in numerous C3 plants is interesting because (a) large quantities of isoprene are emitted, 5×10^{14} g of C annually, (b) a plant may release 1 to 8% of its fixed C as isoprene, and (c) the function of plant isoprene production is unknown. Because of the dependence of foliar isoprene emission on light, the existence of a plastidic isoprene synthase has been postulated. To pursue this idea, a method to isolate chloroplasts from *Salix discolor* was developed and shows a plastidic isoprene synthase that is tightly bound to the thylakoid membrane and accessible to trypsin inactivation. The thylakoid-bound isoprene synthase has catalytic properties similar to known soluble isoprene synthases; however, the relationship between these enzymes is unknown. The discovery of a thylakoid-bound isoprene synthase with a stromal-facing domain places it in the chloroplast, where it may be subject to numerous direct and indirect light-mediated effects. Implications for the light-dependent regulation of foliar isoprene production and its function are presented.

Isoprene (2-methyl-1,3-butadiene) is a volatile hydrocarbon emitted from the leaves of numerous C_3 plant species (Zimmerman, 1979; Guenther et al., 1994). Annual global estimates of foliar isoprene production, 5×10^{14} g of C (Guenther et al., 1995), are similar to those for methane, the most abundant naturally emitted hydrocarbon (Graedel and Crutzen, 1993). Biogenic isoprene emission has a significant impact on tropospheric chemistry, since isoprene is a principal reactant in the formation of tropospheric ozone (Chameides et al., 1988) and an important modulator of the oxidation potential of the troposphere (Brasseur and Chatfield, 1991). From a physiological perspective, foliar isoprene production represents a significant "loss," typically 1 to 8%, of fixed C (Monson and Fall, 1989). This may represent a larger allocation of C than that to a particular defense compound; for example, the damage-induced production of alkaloids in wild tobacco represents a transitory allocation of approximately 1% of fixed C (Baldwin, 1991). Yet the function (if any) of isoprene emission from plants remains unclear (Fall, 1991). Isoprene production may help stabilize plant membranes against high temperatures (Sharkey and Singsaas, 1995), or it may be a mechanism to alter flowering in nearby plants (Terry et al., 1995).

Although foliar isoprene emission was first detected almost 40 years ago (Sanadze, 1957), the biochemistry of its production is just beginning to be elucidated. Isoprene synthesis was first postulated to occur nonenzymatically via the acid-catalyzed conversion of DMAPP to isoprene. Since foliar isoprene emission is light-dependent, this nonenzymatic reaction was thought to occur in the thylakoid lumen of the chloroplast, which undergoes light-induced acidification (reviewed by Sanadze, 1991). Recently, however, the enzyme isoprene synthase, which catalyzes the conversion of DMAPP to isoprene, has been isolated and purified from aspen and velvet bean leaves (Silver and Fall, 1991, 1995; Kuzma, 1995). Foliar isoprene emission appears to be dependent on the activity of this enzyme. For example, the temperature profile of aspen leaf isoprene emission parallels the temperature response of the isolated enzyme (Monson et al., 1992), and isoprene synthase activity parallels leaf isoprene emission over the course of leaf development in velvet bean (Kuzma and Fall, 1993).

Several observations support the existence of a plastidic isoprene synthase: (a) foliar isoprene production requires light; (b) soluble isoprene synthase activity is dependent on $[Mg^{2+}]$ and has an optimum at pH 8 (Silver and Fall, 1991), conditions that occur in the chloroplast stroma in the light; and (c) isolated poplar chloroplasts have been shown to produce isoprene, albeit in low levels (Mgaloblishvili et al., 1981). Our goal, therefore, has been to determine whether a plastidic isoprene synthase exists. We chose to work with willow (*Salix discolor*) because, of the high-isoprene-emitting species tested, chloroplast isolations were most successful using willow leaves. The work presented here localizes an isoprene synthase activity to the plastid and describes a thylakoid-bound form of isoprene synthase. Based on the properties of this enzyme, a model for the light regulation of isoprene production and hypotheses

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Abbreviations: Chl, chlorophyll; DMAPP, dimethylallyldiphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GB, grinding buffer; IPP, isopentenyl diphosphate.

about the function of foliar isoprene production are presented.

MATERIALS AND METHODS

Willow leaves (*Salix discolor*) were collected from a naturally growing population in Boulder, CO. Willow clones from this population were propagated and grown in 10-gallon plastic containers in Metro Mix 350 (American Clay, Denver, CO) and fertilized weekly with Peters Professional Soluble Plant Food—General Purpose Special (Peters Fertilizer Products, Fogelsville, PA). The plants were grown in a greenhouse with supplemental lighting (500 μmol m^{-2} s^{-1}) from low-pressure sodium vapor lamps (General Electric) for a 16-h photoperiod. Temperatures ranged from 21°C (night) to 27°C (day). Willow leaves were taken from the naturally growing population during the summer season and from the greenhouse clones during the rest of the year. Only healthy, mature willow leaves were used.

Reagents

DMAPP was synthesized and purified as previously described (Davisson et al., 1985). Reagents for the synthesis of DMAPP were supplied by Aldrich. The structure of DMAPP was confirmed by TLC and ^{1}H- and ^{31}P-NMR using a 300-MHz Varian VXR-300S instrument (Sunnyvale, CA). Stock solutions of DMAPP were made up in 2 mM ammonium bicarbonate (pH 7) and stored at −20°C. Enzymes and other reagents were purchased from Sigma unless otherwise specified.

Enzymatic Assays

Isoprene Synthase Assays

Isoprene production was assayed in 4.8-mL glass vials sealed with Teflon-lined septa. After a 10-min incubation at 35°C, 1 mL of headspace was analyzed for isoprene by GC with a mercuric oxide detector as described previously (Silver and Fall, 1991; Greenberg et al., 1993). For each sample, background levels of isoprene produced by the nonenzymatic conversion of DMAPP to isoprene were assessed using the procedures described below, but with buffer instead of the plant fraction. All samples were run at least twice within a linear range of activity.

For the chloroplast-enrichment and subplastid-localization experiments, 100 μL of plant sample was incubated in 126.5 μL of final volume with 8 mM $MgCl_2$, 8 mM DMAPP, 0.01 to 0.02% (v/v) Triton X-100 (Sigma), and 2 mM Na vanadate. Total protein in the assay ranged from 0.3 to 0.9 mg. Triton X-100 was included to disrupt subcellular organelles (e.g. chloroplasts), and Na vanadate was included as an inhibitor of phosphatases (Croteau and Karp, 1979).

For the combined chloroplast-, mitochondria-, and peroxisome-enrichment experiment, 100 μL of plant sample (0.2–4.0 mg of protein) was incubated in 118 μL total volume with 0.02% (v/v) Triton X-100, 10 mM DMAPP, and 13 mM $MgCl_2$. The concentrated samples were tested in an attempt to detect activity in fractions with low levels of isoprene synthase.

For the thylakoid-bound isoprene-synthase-solubilization experiments (ionic and chaotropic, and detergent treatments), 100 μL of sample was incubated in 116 to 122 μL of final volume with 10 mM DMAPP and 8 mM $MgCl_2$. Isoprene synthase activities for the sonication experiments were assayed in the same way as for the chloroplast-enrichment experiment above.

Details of the isoprene synthase assays for the experiments examining enzymatic properties of the thylakoid-bound isoprene synthase are included with the protocols for those experiments.

Enzymatic Marker Assays

Enzymatic marker assays were measured at 25°C for all fractions. Inhibition of activity by each plant fraction was examined by spiking the samples with known quantities of enzyme when possible. Assays were optimized so that activities were linear with time for at least 1 min. In addition, activity for each plant fraction was proportional to the quantity of plant fraction added. Assays utilizing the oxidation of NAD(P)H were not suitable due to competing reactions, most probably involving NAD(P)H oxidases. Plant fractions with extremely low levels of activity sometimes gave nonlinear results; their activity was designated "negligible." Background levels were determined by assaying in the absence of substrate unless otherwise indicated. Duplicates were run for each sample and background.

The chloroplast stromal marker NADP-GAPDH was quantified using the method of Ferri et al. (1978). Catalase was assayed spectrophotometrically for use as a peroxisome marker (Luck, 1965). The mitochondrial enzyme Cyt *c* oxidase was measured following the procedure of Ikeda et al. (1994), except that 0.02% Triton X-100 was used to optimize activity (a trade-off between rupturing the mitochondria fully and inhibiting enzymatic activity). The Cyt *c* was reduced with potassium bisulfite until the A_{550}/A_{565} ratio was greater than 6 (Tolbert, 1973). The Cyt *c* oxidase background measurement used the appropriate plant extraction buffer in place of the plant fraction, since the exclusion of the substrate (reduced Cyt *c*) resulted in no absorbance at the given wavelengths. All marker assay absorbance readings were performed using a UV-visible spectrophotometer (UV160U, Shimadzu, Columbia, MD).

Chl and Protein

Chl was determined using 80 or 100% acetone according to the method of Lichtenthaler (1987). Protein concentrations were determined by the Bradford assay (1976) with BSA as the standard protein.

Willow Leaf Fractionation Protocols

Chloroplast-Enrichment Protocol

Willow chloroplasts were isolated using a modified procedure of Falbel and Staehelin (1994). To reduce starch content in the chloroplasts, willow stems were cut the evening prior to use, placed in water, and stored in the dark. They were then illuminated at 500 μmol m^{-2} s^{-1} for

1 h prior to use. Ten grams of leaves were cut, rinsed in distilled water, 2% (v/v) bleach and 0.05% (v/v) Nonidet P-40 (Sigma) detergent solution, and rinsed three to four more times in distilled water to remove surface microorganisms and debris. They were then cut into small pieces (of approximately 2 cm^2) using sharp scissors and directly added to 100 mL of prechilled (on ice) GB1, consisting of 0.5 M sorbitol, 50 mM Tricine (pH 7.8), 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM DTT and 0.1% defatted BSA (Calbiochem), which were added just before use. All solutions and equipment coming into contact with the plant solution were prechilled, and the plant fractions were kept on ice. The leaves were then homogenized using a blender (Waring) with a small volume attachment three times for 2 s each. This solution was filtered through cheesecloth, Miracloth (Calbiochem), and 202-μm (excludes whole cells) and 20-μm (excludes protoplasts) nylon filters (Tekto, Briarcliff Manor, NY). This homogenate is referred to as the "crude." The crude was then centrifuged without braking (HB-4 rotor, DuPont-Sorvall) for 5 min at 2000g. The supernatant is termed "SN1." The pellet, which was enriched for crude chloroplasts, was resuspended with care using a paintbrush in GB1 without BSA in a volume 2% of the original grinding volume, and is designated "P1." P1 was then layered on a 40% Percoll gradient in GB1 without BSA. Centrifugation in a swinging-bucket rotor without braking for 15 min at 7000g resulted in distinct layers, with purified (intact) chloroplasts in the pellet. This pellet was resuspended in the same manner and volume as P1 and termed "P2." Aliquots were removed at each step for analysis, and resuspension volumes were adjusted to compensate. In addition, all total protein, Chl, and enzymatic activities per fraction were adjusted to reflect the removal of these aliquots. Visual inspection at 400 and 1000× with a microscope was used to characterize plant fraction components and to estimate the percentage of intact chloroplasts. Enzymatic estimation of intactness using NADP-GAPDH with and without osmoticum was also employed, particularly in optimizing the chloroplast-isolation protocol.

Combined Chloroplast-, Mitochondria-, and Peroxisome-Enrichment Protocol

The chloroplast enrichment was performed as above with the following modifications. Thirty grams of willow leaves were collected after exposure to a few hours of morning sunlight and ground in 280 to 300 mL of GB2, which consisted of GB1 plus 5 mM $MgCl_2$, 1 mM PMSF, and 1 mM benzamidine HCl. The protease inhibitors were included to limit degradation over the longer time course of this experiment. Enrichment of the mitochondria and peroxisome fractions (Morre et al., 1987) was examined by centrifuging SN1 first at 12,000g for 15 min and resuspending the mitochondrial pellet, followed by centrifugation of this supernatant for 20 min at 18,000g to obtain the peroxisomal pellet. Each of the pellets from this protocol were resuspended in GB2 in a volume 2% of the original GB volume.

Subplastid Localization Protocol

The purified chloroplast fraction, P2, was obtained as described in the chloroplast-enrichment protocol above, but with a fresh-weight-willow-leaves-to-GB-volume ratio of 1:6 (w/v). To remove any residual Percoll associated with P2, P2 was repelleted and the chloroplasts were then ruptured by resuspending in swelling buffer (50 mM Tricine [pH 7.8], 1 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT) in a volume 1% of the original GB for 5 min on ice. More stringent rupturing conditions (e.g. swelling buffer volume 10% of the original GB volume; 30-min exposure time) were also tested. The ruptured P2 solution was then fractionated using sorbitol density gradient separation (0.6/0.93 M steps) similar to Douce and Joyard (1980). Centrifugation for 60 min at 72,000g resulted in three subfractions: the pellet (thylakoid subfraction), a yellow band at the interface of the two layers (envelope membrane subfraction), and the supernatant (soluble subfraction). The thylakoid pellet (P3) was then resuspended in swelling buffer (same volume as for P2). In some cases, P3 underwent an additional wash in swelling buffer. Aliquots of the P2, P3, and the soluble subfraction (S3) were assayed for isoprene synthase activity, NADP-GAPDH activity, Chl, and protein.

Thylakoid-Bound Isoprene Synthase Solubilization Experiments

Willow thylakoids were isolated using a procedure modified from Robinson and Dry (1992). Typically, 30 g of willow leaves were collected after exposure to a few hours of morning sunlight, ground in 280 to 300 mL of GB1 or GB2 (typically GB2), and filtered as above, but without the 20-μm nylon filter. Centrifugation at 6000g for 10 min yielded an enriched chloroplast fraction. To rupture the chloroplasts, this pellet was resuspended in 50 mL (17% of the original grinding buffer volume) of GB2 without sorbitol and centrifuged for 10 min at 6000g. The rupturing procedure was repeated and the thylakoid pellet was resuspended as necessary for a given experiment.

Ionic and Chaotropic Treatments

For these experiments, 1 mM DTT, 1 mM benzamidine HCl, and 1% PEG-400 were included in the rupturing buffer and in the wash solutions to inhibit proteases and aid in retaining active solubilized protein. Equivalent thylakoid pellets were obtained by aliquoting the resuspended thylakoids into the appropriate number of tubes and centrifuging. These pellets were then resuspended in the treatment solutions in a total volume equal to 2% of the original grinding buffer volume. After 10 min on ice, they were centrifuged at 35,000g for 20 min and the pellets were resuspended in the previous volume of GB2. The supernatant solutions were carefully removed. In some cases, an additional wash of the final pellet with GB2 was performed to assess whether any residual treatment solution was inhibiting isoprene synthase activity. Each experiment included a control treatment solution (GB2) to which the results of the other treatment solutions were normalized, as shown in Table I. Wash solutions (Findlay, 1987; Miyake

and Asada, 1992) included 2 mM EDTA, 2 mM Tricine (pH 7.8); 1 M KCl, 50 mM KPi (pH 7.8); 2 M NaBr, 10 mM Tricine (pH 7.8); 100 mM Na carbonate (pH 11); 2 M NaSCN, 10 mM Tricine (pH 7.8); and 8 M urea. The supernatant for the Na carbonate wash was neutralized immediately after centrifugation. Aliquots were removed at each step and isoprene synthase activity and protein were assayed for the fraction of thylakoid pellet resuspended in treatment solution (an indicator of inhibition of activity by the wash solution) and the posttreatment thylakoid and supernatant fractions. In most cases, the supernatant fraction was concentrated using diafiltration and/or ammonium sulfate precipitation in an attempt to detect solubilized activity.

Detergent Treatments

The detergent treatment experiments were performed similarly to those above with the following modifications. Rupturing and detergent solutions did not include 1% PEG-400. After resuspending the individual thylakoid pellets in the detergent solutions to a total volume of 2% of the original GB volume (as above), and removing an aliquot, additional detergent solution was added to each to obtain a solution ratio (μg Chl/mL detergent) of approximately 25. After 10 min on ice, the thylakoids were pelleted as above. Because the supernatants were diluted compared with the thylakoid fractions, attempts to concentrate the supernatant fractions by ammonium sulfate fractionation and diafiltration were undertaken. The detergent solutions were composed of the rupturing solution (which includes 50 mM Tricine [pH 7.8] and 5 mM $MgCl_2$) with the addition of the appropriate detergent: 0.1% deoxycholate, 1.0% 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate, 0.1% Nonidet P-40, 0.1% Triton X-100, or 0.1% Tween-80. Aliquots were removed at each step and isoprene synthase activity, protein, and Chl were assayed for the following fractions: thylakoid pellet resuspended in detergent solution (an indicator of inhibition of activity by the wash solution), thylakoid pellet resuspended in detergent solution to approximately 25 μg Chl/mL detergent solution, the thylakoid and supernatant fractions after detergent treatment, and the concentrated supernatant fractions.

Sonication

The thylakoid fraction P3 was prepared as in the subplastid-localization protocol. Additional swelling buffer was added to 2.5% of original GB volume. The thylakoids were sonicated in 30-s pulses for 2 min (setting 5) using a sonicator (W-225, Heat Systems-Ultrasonics, Farmingdale, NY), centrifuged at 40,000*g* for 20 min, and resuspended in the original volume of P3. The original P3, the supernatant, and pellet fractions were assayed for isoprene synthase activity, Chl, and protein.

Thylakoid-Bound Isoprene Synthase Enzymatic Properties

Mg Ion Dependence

Isolated willow thylakoids were resuspended in 50 mM Tris (pH 8.0), 5% glycerol, 1 mM DTT, and 1 mM benzamidine HCl and dialyzed against this solution for 1 h to remove any residual Mg^{2+}. The dialyzed thylakoid sample was then assayed for isoprene synthase activity with 0.45 mg of thylakoid protein, 10 mM DMAPP, and increasing concentrations of Mg^{2+} (0–25 mM).

pH Dependence

Isolated willow thylakoids were assayed for isoprene synthase activity at pH 5 to 11 in two ways: by adjusting the pH of the isoprene synthase activity assay solution, and by pelleting the thylakoids and resuspending them in solutions of varying pH. The following buffers were utilized as specified: Mes, pH 5.0, 6.0, 7.0; Tricine, pH 7.0, 8.0, 9.0; 2-(*N*-cyclohexylamino)ethanesulfonic acid, pH 9.0, 10.0; and 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.0. Willow thylakoids, resuspended in GB1, were assayed for isoprene synthase activity using 0.29 mg of thylakoid protein (100 μL) with 8 mM $MgCl_2$, 10 mM DMAPP, and approximately 100 mM buffer solution. In additional experiments, pelleted thylakoids were resuspended in buffer solutions containing 50 mM buffer, 5% glycerol, 20 mM $MgCl_2$, and 10 mM DTT. Isoprene synthase was assayed using 0.19 mg of thylakoid protein (100 μL), 10 mM DMAPP, and 8 or 18 mM $MgCl_2$.

Apparent K_m

Isolated willow thylakoids were assayed for isoprene synthase activity using 0.5 mg of thylakoid protein (100 μL), 18 mM $MgCl_2$, and varying concentrations of DMAPP (0–20 mM DMAPP).

RESULTS

Development of a Chloroplast Isolation Procedure

To test for the presence of a plastidic isoprene synthase, the development of a suitable chloroplast system was essential. Unfortunately, we were not able to use well-characterized chloroplast preparations (spinach or pea), since they do not emit isoprene. In addition, chloroplast isolations are generally more successful with chloroplasts from younger leaves; however, due to the developmental dependence of isoprene emission (Kuzma and Fall, 1993), we were required to use mature leaves. Numerous chloroplast isolation methods were pursued with several isoprene-emitting plants. Our best recoveries of intact chloroplasts were from willow leaves using a modified protocol of Falbel and Staehelin (1994). Two important parameters in developing this isolation procedure were (a) the recovery of Chl in the crude and intact chloroplast fractions, an indicator of the success of the plant cell disruption process in retaining chloroplast components, and (b) the degree of chloroplast intactness in the purified chloroplast fraction. Typical values for these parameters were 38 and 30% recovery of Chl in the crude and purified chloroplast fractions, respectively, and 60% intactness in the purified chloroplast fraction. Percentage of intactness was assessed by comparing intact and ruptured chloroplast values for the stromal plastid enzyme NADP-GAPDH, and visually (400 and

1000× magnifications). Other intactness assays (i.e. ferricyanide oxygen evolution and 6-phosphogluconate dehydrogenase activity) were not compatible with the willow system.

Evidence for a Plastidic Isoprene Synthase

As shown in Figure 1, willow isoprene synthase was greatly enriched in the P1 and P2 fractions. The plastid stromal marker NADP-GAPDH also exhibited enriched activity in the chloroplast fractions, particularly in P2, as would be expected. In addition, Chl content per milligram of protein, which may be used as a thylakoid membrane marker, increased in the chloroplast fractions. Isoprene synthase activity was typically below detection levels in the willow crude and SN1 fractions. The inclusion of protease inhibitors (PMSF and benzamidine hydrochloride) and protein stabilizers (1% [v/v] PEG-400 and 1% [w/v] soluble PVP) in the isolation buffer did not result in detectable isoprene synthase activity for these fractions. The addition of the phosphatase inhibitor Na vanadate or the detergent Triton X-100 when assaying for isoprene synthase activity did not yield detectable activity. The supernatant fraction did not appear to contain inhibitors (or to lack activators) of isoprene synthase, since the addition of SN1 to active chloroplast fractions did not alter the production of isoprene by these fractions (data not shown). Attempts to concentrate the SN1 fraction using diafiltration and ammonium sulfate precipitation were unsuccessful, probably due to secondary compounds (such as lipids and polyphenols), which fractionated with SN1. In addition, the lack of substantial activity for prenyl diphosphate-utilizing enzymes in crude fractions has been noted previously (e.g. limonene synthase; Rajaonarivony et al., 1992).

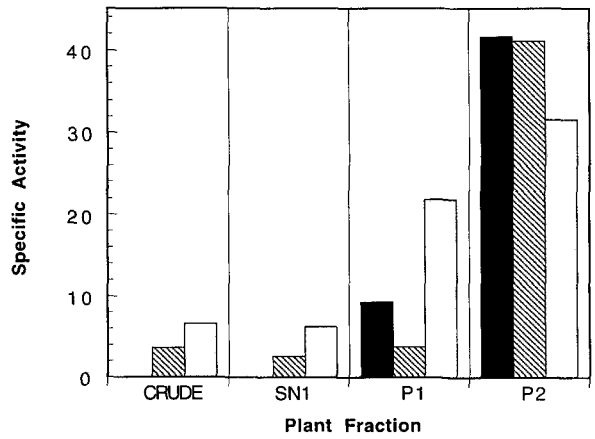

Figure 1. Specific activities of isoprene synthase (pmol isoprene min^{-1} mg^{-1}), NADP-GAPDH (nmol NADP min^{-1} mg^{-1}), and Chl content (μg mg^{-1}) over the course of a willow chloroplast enrichment. Crude willow leaf extract was separated into P1 and SN1 fractions by centrifugation. P2 was then obtained from P1 via Percoll density separation. Enrichment and assay procedures are detailed in "Materials and Methods." The values displayed are from a typical experiment, replicated three times. Black columns, Isoprene synthase; striped columns, NADP-GAPDH; gray columns, Chl.

Therefore, the existence of a soluble willow isoprene synthase (in SN1 fraction) cannot be discounted.

Confirmation of the enzymatic nature of the isoprene production from the crude and purified chloroplast fractions was then explored. To address whether plastidic isoprene production from willow could be nonspecific, chloroplast enrichments (as described for Fig. 1) using spinach, a species that does not emit isoprene, were undertaken. No isoprene synthase activity was associated with any of the spinach fractions (crude, P1, SN1, P2). In addition, treatment of the willow P2 fraction by boiling for 10 min resulted in a total loss of isoprene synthase activity, as did treatment of ruptured P2 with trypsin. These results suggest that a specific enzyme is responsible for plastidic isoprene production.

The plastidic location of this isoprene synthase activity was further verified by assessing contamination of the chloroplast fractions from other plant organelles. The most likely contaminants of the P2 are mitochondria and peroxisomes, based on our chloroplast-isolation procedure, which includes filtration, differential centrifugation, and density gradient centrifugation. To determine if isoprene synthase was associated with these organelles, a combined chloroplast-, mitochondria-, and peroxisome-enrichment experiment was performed. The specific activities of isoprene synthase, Cyt *c* oxidase (mitochondrial marker), catalase (peroxisomal marker), and the Chl content (plastidic marker) of each fraction are presented in Figure 2. Isoprene synthase activity and Chl were enriched in the plastid fractions, and there appeared to be a slight enrichment of isoprene synthase activity and Chl in the mitochondrial pellet. This was likely due to pelleting of chloroplasts remaining in SN1 rather than to a mitochondrial form of the enzyme, since the pattern of isoprene synthase activity enrichment was very different from that of the mitochondrial marker. As expected, Cyt *c* oxidase activity was greatest in the PM fraction and catalase activity was highest in the peroxisome-enriched pellet. In addition, the profiles of these marker enzymes through the plastid enrichment were consistent with expected results. Typical contamination of P2 by mitochondria and peroxisomes as a percentage of their total enzyme marker activity was 2 and 10%, respectively. Recoveries of the plastidic markers Chl and NADP-GAPDH in the P2 fraction were typically 30% of total activity. Thus, the isoprene synthase activity is indeed plastidic and is not due to contamination from other plant organelles, particularly mitochondria and peroxisomes.

Evidence for a Thylakoid-Bound Isoprene Synthase

Subplastid localization experiments were designed so that the thylakoid membranes could be separated from soluble stromal and envelope membrane fractions. Results are shown in Figure 3. Isoprene synthase activity remained associated with the thylakoid membrane fraction P3, as did Chl. In contrast, NADP-GAPDH specific activity was greatly enhanced in the stromal fraction S3. Isoprene synthase activity was not detected in S3 or in 30-fold-concentrated S3 (ammonium sulfate precipitation followed by resuspension and dialysis). Thylakoid isola-

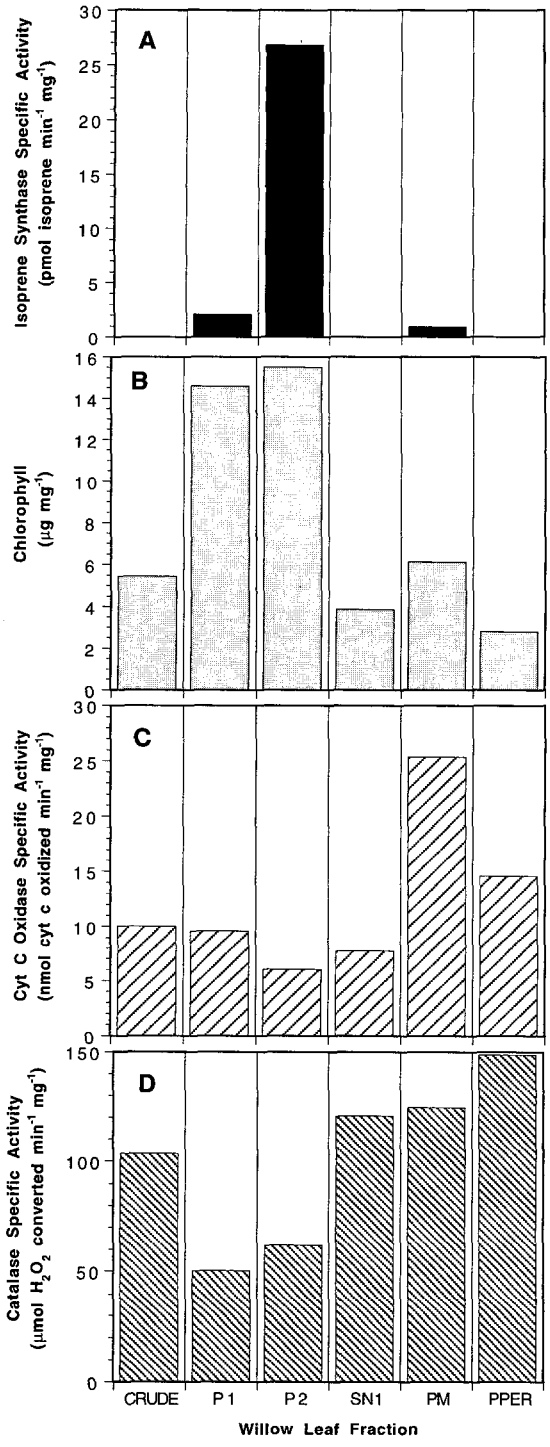

Figure 2. Fractionation of willow leaf isoprene synthase (A), the plastidic marker Chl (B), the mitochondrial marker Cyt *c* oxidase (C), and the peroxisomal marker catalase (D) through the course of a combined chloroplast-, mitochondria-, and peroxisome-enrichment protocol. Crude willow leaf extract was fractionated into P1 and SN1 fractions. A purified chloroplast fraction was then obtained from P1 via Percoll density separation. Further centrifugation of SN1 resulted in mitochondrial (PM) and peroxisomal (PPER) pellets. Enrichment and assay protocols are detailed in "Materials and Methods." The results shown are from a typical experiment, repeated several times.

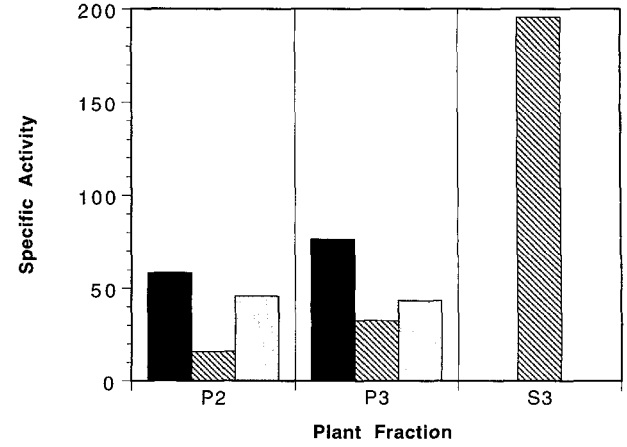

Figure 3. Subplastid localization of isoprene synthase in willow. Specific activities for isoprene synthase (10 pmol isoprene min^{-1} mg^{-1}), NADP-GAPDH (nmol min^{-1} mg^{-1}), and Chl content (μg mg^{-1}) are shown for a typical experiment, replicated several times. The P2 was ruptured and separated into thylakoid (P3) and stromal (S3) fractions. Localization and assay procedures are given in "Materials and Methods." Black columns, Isoprene synthase; striped columns, NADP-GAPDH; gray columns, Chl.

tions were performed under a number of different conditions, including (a) with and without protease inhibitors, (b) with 1 and 5 mM $MgCl_2$, (c) with and without 1% (v/v) PEG-400 (a protein stabilizer), and (d) under more stringent P2 rupturing conditions. In each case, isoprene synthase activity remained associated with the thylakoid membranes. Typical contamination of P3 by mitochondria (as assessed by Cyt *c* oxidase activity) and peroxisomes (determined by catalase activity) as a percentage of their total activity was 0 and 4%, respectively. Recovery of the thylakoid marker Chl in the P3 fraction was typically 22% of total Chl content.

An electron micrograph of a typical P3 preparation is shown in Figure 4. As illustrated, the thylakoid fraction contained primarily stacked thylakoid grana. This result supports the localization of the plastidic isoprene synthase to the thylakoid membranes.

Membrane Association of the Thylakoid-Bound Isoprene Synthase

Is the thylakoid-bound isoprene synthase a peripheral membrane protein, an integral membrane protein, or a lumenal protein? A series of experiments designed to answer these questions was undertaken. Table I displays isoprene synthase activity and total protein associated with the thylakoid membrane and solubilized (supernatant) fractions following various solubilization procedures.

The release of peripheral membrane proteins via disruption of ionic, H-bonding, and hydrophobic interactions was first attempted. Isoprene synthase activity remained associated with the thylakoid membranes following vigorous vortexing and treatment with (a) 2 mM EDTA, 2 mM Tricine (pH 7.8); (b) 1 M KCl, 50 mM KPi (pH 7.8); or (c) 2 M NaBr, 10 mM Tricine (pH 7.8). It appears, therefore, that metal cations and ionic interactions are not the principal partici-

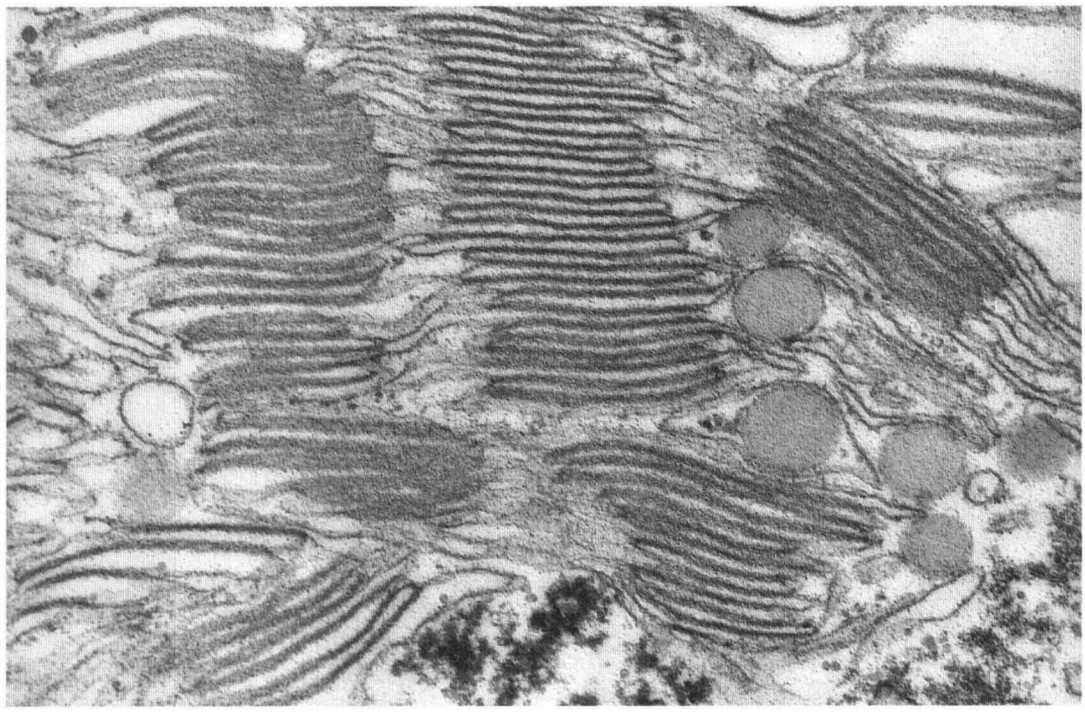

Figure 4. Electron micrograph of isolated willow thylakoids. Willow thylakoids (P3 fraction) were prepared as in Figure 3. For electron microscopy, the thylakoid pellet was fixed with glutaraldehyde and postfixed with OsO_4. Embedding was performed by gradually introducing EPON-Araldite (Ted Pella, Redding, Ca) in propylene oxide. Thin sections were poststained with uranyl acetate and Reynold's lead. Magnification = 33,000×.

pants in binding isoprene synthase to the thylakoid membrane and that isoprene synthase is not a loosely bound peripheral membrane protein. More stringent treatments that interfere with both ionic- and hydrogen-bonding interactions (100 mM Na carbonate, pH 11), or with hydrophobic interactions (2 M NaSCN, 10 mM Tricine, pH 7.8, or 8 M urea), dramatically decreased isoprene synthase activity associated with the thylakoid membranes. However, all three of these treatment solutions inhibited isoprene synthase activity as shown by the lack of activity in the thylakoid fraction with treatment solution prior to centrifugation. Including a posttreatment wash of the thylakoid pellets in GB (to remove any residual treatment solution) did not increase their isoprene synthase activities. Solubilized active enzyme was not detected for any of the above treatments. Concentration and resuspension of the solubi-

Table I. *Attempts to solubilize willow thylakoid-bound isoprene synthase*

Solubilization treatments of the willow thylakoid fraction (P3) are described in "Materials and Methods." Values presented are the average of two or three separate experiments. Detergent interference with the Bradford method of protein quantitation accounts for the high protein concentrations present in some of the supernatant fractions. CHAPSO, 3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DOC, deoxycholate; NP-40, Nonidet P-40; TX-100, Triton X-100; TW-80, Tween-80.

Solubilization Treatment	Percent of Original Isoprene Synthase Activity Associated with Each Fraction		Percent of Original Protein Associated with Each Fraction	
	Thylakoid	Supernatant	Thylakoid	Supernatant
Ionic and chaotropic treatments				
2 mM EDTA/2 mM Tricine (7.8)	88	0	102	22
1 M KCl/50 mM KPi (7.8)	120	0	90	6
2 M NaBr/10 mM Tricine (7.8)	66	0	71	6
100 mM Na carbonate (11.0)	6	0	41	167
8 M Urea	26	0	67	18
2 M NaSCN/10 mM Tricine (7.8)	0	0	67	69
Detergent treatments				
1.0% CHAPSO (zwitterionic)	10	0	64	70
0.1% DOC (anionic)	52	0	96	17
0.1% NP-40 (nonionic)	26	0	59	149
0.1% TX-100 (nonionic)	28	0	65	133
0.1% TW-80 (nonionic)	48	0	119	65

lized (supernatant) fractions in control buffer also did not result in detectable isoprene synthase activity. Therefore, the ability of these treatments to solubilize the enzyme are inconclusive, since we were limited to detection of isoprene synthase activity, not the protein.

Detergent treatments that could solubilize integral membrane proteins were then employed. Treatments included the anionic detergent deoxycholate, the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate, and the nonionic detergents Nonidet P-40, Triton X-100, and Tween-80. Significant isoprene synthase activity remained associated with the thylakoids following treatment with 0.1% deoxycholate and 0.1% Tween-80, supporting a tightly bound isoprene synthase. The other detergent treatments were inconclusive in that little isoprene synthase activity remained in the thylakoid fraction, yet active solubilized enzyme was not detected (even after concentration) for any of these treatments.

To determine whether isoprene synthase is a lumenal protein, sonication, freeze-thawing, and trypsin digestion of the thylakoids were performed. Activity remained with the membranes following sonication and freeze-thawing of the P3 thylakoid fraction (data not shown). In addition, trypsin digestion experiments were performed by incubating ruptured purified willow chloroplasts (P2) at 30°C with 1.6 mg/mL trypsin, followed by the addition of 8 mg/mL trypsin inhibitor. In the control samples, trypsin inhibitor was added prior to the addition of the trypsin. Within 10 min, isoprene synthase activity was reduced 92% by trypsin treatment. This result indicates that thylakoid-bound isoprene synthase is exposed to the chloroplast stroma and, therefore, is not a lumenal protein.

Although active isoprene synthase was not solubilized by any of these treatments, the results do provide clues as to the nature of the isoprene synthase-thylakoid membrane association and also provide techniques useful for the future purification of the thylakoid-bound enzyme. Based on the above results, isoprene synthase appears to be tightly bound to the thylakoid membrane with a domain that probably contains the active site exposed to the chloroplast stroma.

Enzymatic Properties of Thylakoid-Bound Isoprene Synthase

Some of the enzymatic characteristics of thylakoid-bound isoprene synthase were examined and compared with the soluble forms of the isoprene synthase enzyme isolated previously from whole aspen or velvet bean leaves ground in liquid nitrogen (Silver and Fall, 1991, 1995; Kuzma and Fall, 1993; Kuzma, 1995). These properties are summarized in Table II. It should be noted that the whole-leaf extraction procedure utilized in isolating the soluble isoprene synthase enzymes from aspen and velvet bean leaves was not successful for willow leaves. Concentration of the crude extract by ammonium sulfate precipitation was ineffective (likely due to large amounts of secondary compounds in willow leaves); a whole-leaf extraction procedure for willow is currently being developed.

Table II. *Comparison of characteristics of thylakoid-bound and soluble forms of isoprene synthase*

Willow thylakoid-bound isoprene synthase experiments are detailed in "Materials and Methods." Aspen soluble isoprene synthase data are reported by Silver and Fall (1991, 1995). Velvet bean soluble isoprene synthase values are from Kuzma and Fall (1993) and Kuzma (1995).

Enzymatic Characteristic	Willow Thylakoid-Bound Isoprene Synthase	Aspen Soluble Isoprene Synthase	Velvet Bean Soluble Isoprene Synthase
Mg ion optimum	10–15 mM	10–15 mM	15 mM
K_m (DMAPP)	8 mM	8 mM	9 mM
pH optimum	10	8	8

Thylakoid isoprene synthase was found to be dependent on Mg^{2+} or Mn^{2+} for activity. This requirement was specific in that Ca^{2+} did not stimulate activity and was consistent with the soluble isoprene synthases and with other enzymes that use prenyl diphosphate substrates (Alonso and Croteau, 1993). In addition, the Mg^{2+} profile from 0 to 25 mM $MgCl_2$ and optimum (10–15 mM) for thylakoid-bound isoprene synthase were similar to those of the soluble isoprene synthases (Table II).

The apparent K_m of thylakoid isoprene synthase with respect to DMAPP was 8 mM; it was assessed at pH 8 since a domain of the thylakoid-bound isoprene synthase faces the stroma, which undergoes light-dependent alkalization to approximately pH 8. This K_m is similar to that of the soluble isoprene synthases.

The pH optimum (pH 10, Table II) of the thylakoid-bound isoprene synthase was quite different from that of the soluble isoprene synthases (optima at pH 8). However, it should be noted that the pH optima for the soluble isoprene synthases is fairly broad, and that these enzymes show considerable activity at pH 10: 68% of maximal activity for velvet bean isoprene synthase (Kuzma and Fall, 1993). The pH profiles were performed so that different buffers overlapped at given pH values; this minimized any specific buffer or titration effects due to the addition of KOH or NaOH. Whether monovalent cations stimulate isoprene synthase activity, as is the case for certain monoterpene cyclases (Savage et al., 1994), has not been determined. Additional studies (data not shown) indicated that thylakoid-bound isoprene synthase activity at pH 10 was consistently higher than its activity at pH 8 over a range of DMAPP concentrations (0–20 mM DMAPP) and at different Mg^{2+} concentrations (5, 8, 10, and 18 mM $MgCl_2$). Thylakoids typically maintain their negative surface electrical charge over the pH range tested, as shown by electrophoretic mobility studies (Barber, 1988), and should remain stacked. In addition, DMAPP may be partially hydrolyzed to its monophosphate above pH 9 (Davisson et al., 1986); however, this would likely result in a lower (not higher) isoprene production rate. Thus, the pH optimum of 10 for willow thylakoid-bound isoprene synthase appears to be real.

DISCUSSION

Discovery of a Thylakoid-Bound Isoprene Synthase

An isoprene synthase activity was localized to the chloroplasts of *S. discolor* (Fig. 1). This activity was shown to be specific in that chloroplasts from spinach (which does not emit isoprene) did not exhibit isoprene synthase activity. In addition, boiling and trypsin treatment of the willow plastidic fractions eliminated the enzyme activity. Further exploration of the subplastid location of isoprene synthase led to the discovery of a thylakoid-bound isoprene synthase in *S. discolor*, which appears to be tightly bound to the thylakoid membrane, since a variety of solubilization treatments did not release active enzyme (Table I). In addition, it is trypsin-sensitive, which is indicative of a stromal-facing domain.

Initial characterization of the willow thylakoid-bound isoprene synthase suggests that it has catalytic properties similar to the previously isolated soluble isoprene synthases, but with a different pH optimum (Table II). The pH optimum (10) for the thylakoid-bound isoprene synthase is not likely to be of physiological relevance, however, since the chloroplast stroma has not been found to over-alkalinize beyond approximately pH 8.5 (e.g. Wagner et al., 1990). Under conditions occurring in the illuminated plastid stroma (e.g. pH 8, 8 mM Mg^{2+}), enzymatic isoprene production from willow thylakoids (at saturating [DMAPP]) can account for willow leaf isoprene production (assayed at a saturating light intensity of 1000 μmol m^{-2} s^{-1}). Similarly, soluble isoprene synthase activities from aspen and velvet bean (using comparable assay conditions) can account for their foliar isoprene emissions (e.g. Kuzma and Fall, 1993). Because the K_m for both the thylakoid-bound and soluble isoprene synthases are quite high (approximately 8 mM), saturating [DMAPP] may not accurately reflect in vivo conditions. However, the levels of DMAPP in plant compartments are unknown, and it is possible that there are more active forms of the isoprene synthases in vivo.

It remains to be determined if leaves of these different plant species contain both thylakoid-bound and soluble forms of isoprene synthase. It is possible that the soluble form is actually the thylakoid-bound isoprene synthase, with solubilization occurring as an artifact of the whole-leaf extraction process. Alternatively, isoprene synthase could be variably anchored to the thylakoid membrane or there could be two gene products yielding soluble and thylakoid-bound isozymes. The relationship between the thylakoid and soluble forms of isoprene synthase requires further study.

Model for the Light-Dependent Regulation of Foliar Isoprene Production

The discovery of a stromal-facing, thylakoid-bound isoprene synthase, as discussed above, places the enzyme where it can be regulated by direct and indirect light-mediated effects. It has been known for decades that the production of isoprene from leaves requires sunlight (discussed by Sanadze, 1991); we have shown that the response is very rapid. Upon exposure to light, isoprene production occurs almost immediately and maximal isoprene emission is achieved within 20 to 30 min. Withdrawal of light results in an immediate decline in isoprene production, with emissions dropping to zero within a few minutes (Monson et al., 1991). Regulation of isoprene emission by light does not appear to be controlled by stomatal opening (Fall and Monson, 1992), but is likely due to (a) light-dependent activation of isoprene synthase and/or (b) light-dependent supply of the substrate DMAPP. Acid-catalyzed, nonenzymatic conversion of DMAPP to isoprene may occur in the thylakoid lumen, as Sanadze postulated (1991); however, the presence of DMAPP in the lumen has not yet been established.

Our model for the light-dependent regulation of isoprene production in the chloroplast is shown in Figure 5. Plausible mechanisms for the light-dependent activation of the enzyme include pH/Mg^{2+} effects, thioredoxin activation, reversible anchoring, or a phosphorylation event. As mentioned above, when illuminated the chloroplast stroma undergoes alkalization and Mg^{2+} concentration increases, resulting in increased activity for many plastidic enzymes (e.g. plastidic Fru-1,6-bisphosphatase; Baier and Latzko, 1975). Similar changes in vitro can account for a 4-fold increase in willow thylakoid isoprene synthase activity (data not shown). Many light-dependent enzymes are regulated by redox modulation via the thioredoxin system (Scheibe, 1991). Photoreduced chloroplastic thioredoxin can directly reduce disulfide groups within target enzymes, causing conformational changes or making reduced Cys available for catalysis. Reversible anchoring to or association with the thylakoid membrane is also a possible mode of light-dependent regulation, as it is for the thylakoid-associated 32-kD D1 polypeptide (Mattoo and Edelman, 1987) and violaxanthin de-epoxidase (Hager and Holocher, 1994). In addition, light-mediated phosphorylation events have been shown to rapidly regulate thylakoid protein activities (e.g. Elich et al., 1992) and may be involved in thylakoid isoprene synthase activation.

The supply of the substrate DMAPP may also be light-regulated. Given the relatively high K_m of thylakoid isoprene synthase with respect to DMAPP, a light-dependent increase in DMAPP concentration in the chloroplast stroma could greatly influence isoprene production. DMAPP is almost certainly derived from IPP via the enzyme IPP isomerase; however, the subcellular location(s) of IPP (and DMAPP) synthesis in plants is not yet well understood (reviewed by McGarvey and Croteau, 1995). A light-mediated change in DMAPP concentration could be attributed to a light-dependent increase in the flux of fixed photosynthate through a plastid mevalonate acid or alternative pathway (e.g. Schwender et al., 1995), resulting in increased stromal IPP (and DMAPP) concentrations. IPP isomerase activity could also be light-regulated, resulting in increased DMAPP levels. For example, after 3 h of illumination, a 3-fold increase in IPP isomerase activity was observed in conjunction with the light-stimulated transformation of maize etioplasts to chloroplasts (Albrecht and Sandmann, 1994). Alternatively, DMAPP concentrations in the chloroplast stroma could be controlled by the

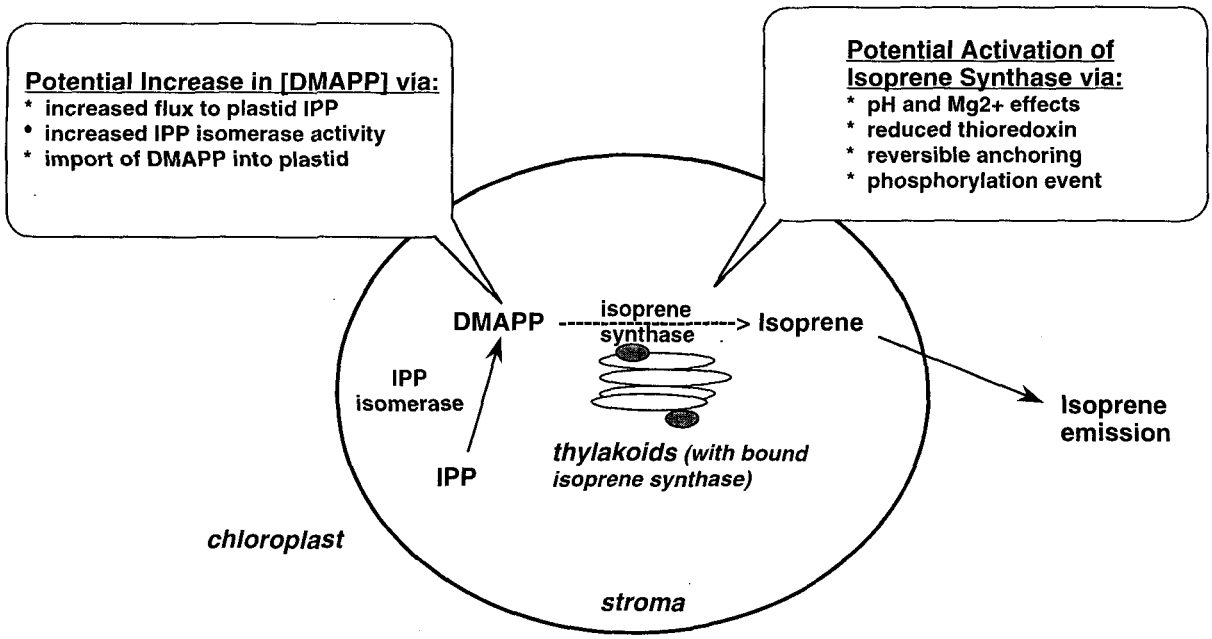

Figure 5. Model for the light-dependent production of isoprene in chloroplasts. Isoprene synthase is tightly bound to the stromal face of thylakoid membranes, where it may be subject to numerous direct and indirect light-mediated effects. These effects likely modulate the activity of the thylakoid-bound isoprene synthase and/or the supply of the substrate DMAPP. Possible mechanisms for this light-dependent regulation are discussed in the text.

light-dependent import of DMAPP (or IPP) from the cytosol to the stroma. Plastidic IPP importers have been reported, and regulation and specificity of import is under investigation (Heintze et al., 1990; Soler et al., 1993).

Implications for Why Plants Produce Isoprene

The discovery of a thylakoid-bound, stromal-facing, isoprene synthase may shed light on the physiological function of isoprene production. Three likely reasons for a thylakoid-bound enzyme include (a) proximity to a membrane-associated substrate, (b) exposure to membrane-associated activators, or (c) product release to the thylakoid membrane and its associated proteins and pigments. Violaxanthin de-epoxidase associates with the thylakoid membrane when active in order to interact with its membrane-soluble substrate violaxanthin (Hager and Holocher, 1994). However, in the case of isoprene synthase, its substrate, DMAPP, is unlikely to be membrane-soluble. Interaction with membrane-associated activators could account for a thylakoid-bound isoprene synthase, since light activation by the thioredoxin system or a protein kinase/phosphatase is possible (discussed above).

Thylakoid-bound isoprene synthase may also be positioned to be able to release the hydrophobic isoprene molecule directly into the thylakoid membrane. This could support several possible functions of foliar isoprene production. Sharkey and Singass (1995) proposed that isoprene serves to protect plant membranes against heat stress (which is presumably greater in the light). The release of isoprene into the thylakoid membrane could greatly increase its effective concentration in the membrane. In addition, various investigators have hypothesized that isoprene could act as an antioxidant or anti-free radical agent. Oxidants and free radicals are photoproduced at the thylakoids. Therefore, the isoprene produced would be nicely situated to react with these species, although it is not clear how the resulting reactive products would be quenched (Elstner, 1987). Alternatively, isoprene released into the thylakoid membrane could serve as a substrate or effector for a thylakoid protein in a yet-unknown reaction.

In conclusion, the isolation and characterization of a thylakoid-bound isoprene synthase gives us a framework for predicting and testing the light-dependent regulation of isoprene emission and for addressing the looming question of why plants produce isoprene.

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